DESCRIPTION

TITLL OF THE INVENTION

NOVEL CENTROMERIC PROTEIN SHUGOSHIN

Technical Field

[0001]

The present invention relates to a protector protein Sgo1 (shugoshin) of cohesin Rec8 derived from fission yeast Schizosaccharomyces pombe, its homologue and paralogue having a regulatory activity of chromosome segregation, and DNAs encoding them.

Background Art

[0002]

In eukaryotes, sister chromatid cohesion is established during S phase of cell cycle and maintained throughout G2 until M phase. During mitosis, this cohesion is destroyed along the entire length of chromosome, allowing sister chromatid to segregate to the opposite sides of cell (equational division) and ensuring that each daughter cell receives one copy of each chromosome. In contrast, meiosis consists of two rounds of chromosome segregation following a single round of DNA replication, leading to the formation of four haploid gametes from one diploid germ cell. During meiosis I, homologous chromosomes (homologues) pair to recombine, forming chiasmata in which one sister chromatid from one homologue is covalently attached to a sister chromatid from the other homologue. Hence, in order for homologues to segregate at meiosis I, cohesion of sister chromatid is necessary to be dissociated along the

chromosome arms to resolve chiasmata. However, sister chromatid cohesion is retained at centromere until meiosis II, and utilizes the residual centromeric cohesion when sister chromatid segregates, in the same manner as it does in mitosis. Thus, meiotic division requires sister chromatid cohesion to be dissociated in two steps. However, the molecular mechanism for protection of centromeric cohesion only during meiosis I and only at the centromere has remained to be elucidated (e.g., see Annu Rev Genet 35, 673-745(2001)).

[0003]

There are important clues as to the molecular nature of sister chromatid cohesion, and the mechanism dissociating sister chromatid cohesion at the onset of anaphase (e.g., see Annu Rev Genet 35, 673-745(2001); Curr Opin Cell Biol 12, 297-301(2000); Curr Biol 13, R104-14 (2003); Annu Rev Cell Dev Biol 17, 753-77(2001); Genes Dev 16, 399-414(2002)). In various eukaryotes, sister chromatid cohesion depends on a multisubunit cohesin complex including Scc1 (Rad21 in fission yeast Schizosaccharomyces pombe). Anaphase promoting complex (APC)-dependent degradation of the securin, Cut2/Pds1, allows to dissociate the Cut1/Esp1 endopeptidase (separase), which in turn cleaves Rad21/Scc1, dissociating sister chromatid cohesion. During meiosis, the cohesion subunit Rad21/Scc1 is replaced with a meiotic counterpart, Rec8 (e.g., see Cell 98, 91-103(1999); Mol. Cell. Biol. 19, 3515-3528(1999); Nature 400, 461-4(1999); Genes Dev 15, 1349-60(2001); J Cell Biol 160, 657-70(2003)). As Rec8 complex resides only at centromere after meiosis I and the depletion of Rec8 destroys centromeric cohesion, the presence of Rec8 at centromere has been thought to confer the persistence of cohesion throughout meiosis I (e.g.,

see Nat Cell Biol 1, E125-7 (1999)). Several lines of evidence suggest that Rec8 along chromosome arms is cleaved by separase at anaphase I while centromeric Rec8 is specifically protected until metaphase II (e.g., see Cell 103, 387-98(2000); Embo J 22, 5643-53(2003)). Budding yeast SPO13 has been implicated in the protection of centromeric Rec8 (e.g., see Genes Dev 16, 1659-71(2002); Genes Dev 16, 1672-81(2002)), but SPO13 is not centromeric and may function indirectly. Drosophila MEI-S332 is a protein residing at centromere, is required for the persistence of centromeric cohesion during meiosis I, and has features of a candidate protector of meiotic centromeric cohesion, although the details of such protection have so far not been elucidated (e.g., see Annu Rev Cell Dev Biol 17, 753-77(2001); Cell 83, 247-256(1995)). Despite the completion of genome sequencing projects on several organisms, no homologue of these proteins has emerged, preventing the formulation of a generalized view of the protection. Concurrently, studies in fission yeast have illuminated the importance of pericentromeric heterochromatin for recruiting centromeric Rec8 complexes and ensuring centromeric cohesion during meiosis I (e.g., see Science 300, 1152-5(2003)). However, pericentromeric heterochromatin cannot alone confer the specific protection of Rec8 at meiosis I toward meiosis II.

Disclosure of the Invention

[0004]

Almost all the eukaryotes including human propagate offsprings by sexual reproduction evolutionarily predominant with a mixture of genome. Meiosis that reduces chromosome number in half is a core part of the sexual reproduction

mechanism. In somatic mitosis, two kinetochores of sister chromatid are caught by spindle microtuble extended from the opposite poles, and sister chromatid is evenly segregated to the both poles by concurrently dissolving the cohesion of arms and centromeres (equational division). In contrast, in meiosis I kinetochores of sister chromatids are caught by spindle microtuble extended from the same pole, and segregated to the same pole while retaining the cohesion at centromere (meiotic division). Next, for the first time in meiosis II the cohesion of centromere site of sister chromatid is dissolved, and separated toward one pole or the other of the two poles respectively, which culminates in the generation of accurate four haploid gametes. Meiosis-specific meiotic division is a modality of chromosome segregation conserved in almost all the eukaryotes, from yeast to human, however regulatory mechanism at the molecular level has remained enigmatic for a long time. The present inventor has demonstrated that meiosis-specific chromosome cohesion factor, cohesin plays an essential role in this regulation by using fission yeast (Nature 400, 461-4(1999); Science 300, 1152-5(2003); Nature 409, 359-363 (2001)). An object of the present invention is to provide meiosis-specific novel kinetochore protein Sgo1 (shugoshin) derived from fission yeast Schizosaccharomyces pombe, and a homologue or paralogue thereof having a regulatory activity of chromosome segregation; and DNAs encoding them; as a factor ensuring the retention of unidirection and cohesion in sister centromere at meiosis I in cooperation with cohesin.

[0005]

Meiosis comprises two steps of specialized nuclear divisions for producing haploid gametes. To accomplish this,

sister chromatid cohesion is necessary to be dissociated in a stepwise manner, first from chromosome arms at anaphase I and second from centromeres at anaphase II. In particular, the factors that protect centromeric cohesion during meiosis I have heretofore remained undissolved. To elucidate the proteins protecting Rec8 during anaphase, the present inventor screened in fission yeast genes for a gene that inhibits mitotic growth and prevents sister chromatid from the separation at anaphase, when co-expressed with Rec8. In this approach, meiosis-specific protein that is a protector of Rec8 in fission yeast and protects (Shugo) centromeric Rec8 from the degradation at anaphase I was indentified, and named Sgo1 (Shugoshin, a Japanese for "guardian spirit"). It was also identified that shugoshin plays an important role in mitotic chromosome segregation, and then identified a budding yeast Sgo1 homologue and a fission yeast mitotic paralogue Sgo2. A marginal similarity between Sgo1 and Drosophila MEI-S332 was identified, and Sgo1 homologue in other eukaryotes was also identified. Shugoshin-like proteins in animal cells, which were predicted from the sequence, also have functional conservation with yeast shugoshin. The present invention has been thus completed based on this knowledge.

[0006]

That is, the present invention relates to (1) a DNA encoding a following protein (a) or (b): (a) a protein consisting of an amino acid sequence shown in SEQ ID NO: 2, (b) a protein comprising an amino acid sequence where one or several amino acids are deleted, replaced or added in an amino acid sequence shown in SEQ ID NO: 2, and having a regulatory activity of chromosome segregation; (2) a DNA consisting of a base

sequence shown in SEQ ID NO: 1 or a complementary sequence thereof; (3) a DNA containing part or whole of a base sequence shown in SEQ ID NO: 1 or a complementary sequence thereof, and encoding a protein that has a regulatory activity of chromosome segregation; (4) a DNA hybridizing with the DNA according to "2" under stringent conditions and encoding a protein that has a regulatory activity of chromosome segregation; (5) a protein consisting of an amino acid sequence shown in SEQ ID NO: 2; and (6) a protein consisting of an amino acid sequence where one or several amino acids are deleted, replaced or added in an amino acid sequence shown in SEQ ID NO: 2, and having a regulatory activity of chromosome segregation.

[0007]

The present invention also relates to (7) a DNA encoding a following protein (a) or (b): (a) a protein consisting of an amino acid sequence shown in SEQ ID NO: 4, (b) a protein consisting of an amino acid sequence where one or several amino acids are deleted, replaced or added in an amino acid sequence shown in SEQ ID NO: 4, and having a regulatory activity of chromosome segregation; (8) a DNA consisting of a base sequence shown in SEQ ID NO: 3 or a complementary sequence thereof; (9) a DNA containing part or whole of a base sequence shown in SEQ ID NO: 3 or a complementary sequence thereof, and encoding a protein that has a regulatory activity of chromosome segregation; (10) a DNA hybridizing with the DNA according to "8" under stringent conditions and encoding a protein that has a regulatory activity of chromosome segregation; (11) a protein consisting of an amino acid sequence shown in SEQ ID NO: 4; and (12) a protein consisting of an amino acid sequence where one or several amino acids are deleted, replaced or added in an amino

acid sequence shown in SEQ ID NO: 4, and having a regulatory activity of chromosome segregation.

[0008]

The present invention further relates to (13) a DNA encoding a following protein (a) or (b): (a) a protein consisting of an amino acid sequence shown in SEQ ID NO: 6, (b) a protein consisting of an amino acid sequence where one or several amino acids are deleted, replaced or added in an amino acid sequence shown in SEQ ID NO: 6, and having a regulatory activity of chromosome segregation; (14) a DNA consisting of a base sequence shown in SEQ ID NO: 5 or a complementary sequence thereof; (15) a DNA containing part or whole of a base sequence shown in SEQ ID NO: 5 or a complementary sequence thereof, and encoding a protein that has a regulatory activity of chromosome segregation; (16) a DNA hybridizing with the DNA according to "14" under stringent conditions and encoding a protein that has a regulatory activity of chromosome segregation; (17) a protein consisting of an amino acid sequence shown in SEQ ID NO: 6; and (18) a protein consisting of an amino acid sequence where one or several amino acids are deleted, replaced or added in an amino acid sequence shown in SEQ ID NO: 6, and having a regulatory activity of chromosome segregation.

[0009]

The present invention still further relates to (19) a DNA encoding a following protein (a) or (b) that has a regulatory activity of chromosome segregation: (a) a protein consisting of an amino acid sequence shown in SEQ ID NO: 8, 10, 12, 14, 16, 18 or 20, (b) a protein consisting of an amino acid sequence where one or several amino acids are deleted, replaced or added in an amino acid sequence shown in SEQ ID NO: 8, 10, 12, 14,

16, 18 or 20; (20) a DNA consisting of a base sequence shown in SEQ ID NO: 7, 9, 11, 13, 15, 17 or 19 or a complementary sequence thereof, and encoding a protein that has a regulatory activity of chromosome segregation; (21) a DNA containing part or whole of a base sequence shown in SEQ ID NO: 7, 9, 11, 13, 15, 17 or 19 or a complementary sequence thereof, and encoding a protein that has a regulatory activity of chromosome segregation; (22) a DNA hybridizing with the DNA according to "7", "9", "11", "13", "15", "17" or "19" under stringent conditions and encoding a protein that has a regulatory activity of chromosome segregation; (23) a protein consisting of an amino acid sequence shown in SEQ ID NO: 8, 10, 12, 14, 16, 18 or 20, and having a regulatory activity of chromosome segregation; and (24) a protein consisting of an amino acid sequence where one or several amino acids are deleted, replaced or added in an amino amino acid sequence shown in SEQ ID NO: 8, 10, 12, 14, 16, 18 or 20, and having a regulatory activity of chromosome segregation.

[0010]

Furthermore, the present invention relates to (25) a fusion protein in which the protein according to "5", "6", "11", "12", "23" or "24" is bound with a marker protein and/or a peptide tag; (26) an antibody specifically binding to the protein according to "5", "6", "11", "12", "23" or "24"; and (27) the antibody according to "26", which is a monoclonal antibody.

Brief Description of Drawings

[0011]

Figure 1 is a set of pictures showing that sister chromatids are not segregated during mitosis by co-expression

of Sgo1 and Rec8 in the present invention. a.) The cen2-GFP strains expressing the genes indicated by endogenous promoters (a constitutive chromatin promoter for rad21+ or rec8+, and a thiamine-repressible promoter Pnmt1 for Sgo1+) were streaked on a thiamine-depleted plate. b.) Samples of Padh1-rec8+ Pnmt1-sgo1+ cells cultured for 15 hours at 30°C after thiamine depletion. The non-segregation of cen2-GFP (asterisk) was identified in the septate junction cells. c.) The non-segregations of cen2-GFP were counted (n>100). d.) The Padh1-rec8+-GFP strains were cultured with or without the use of Pnmt1-sgo1+ in the same manner as (b). Samples of cells at interphase and anaphase are shown.

Figure 2 is a set of pictures showing that sister chromatid segregation was undergone in mitosis by expression of non-cleavable Rec8. The plasmid pREP41-rec8- RDRD (expressing non-cleavable Rec8 (Embo J 22, 5643-53(2003))) was integrated into the chromosome of cen2-GFP cell strains (+Rec8-RDRD), and the cells were streaked on plates with or without the presence of thiamine. The host strain cells (-Rec8-RDRD) were similarly cultured as a control. Note that Rec8-RDRD is expressed only on the thiamine-free plate. Samples of cells cultured in culture medium for 15 hours at 30°C after the depletion of thiamine.

Figure 3 is a set of pictures showing that sgo1 of the present invention is required to protect Rec8 and thereby cohesion at centromeres arises during anaphase of meiosis I. a.) As for one of the homologues marked with cen2-GFP, segregation during meiosis was observed in wild-type and sgo1Δ cells (n>170). A normal segregation pattern of cen2-GFP is illustrated (left). Samples of sgo1Δ cells are shown (right).

b.) Separation of sister cen2-GFP dots after meiosis I (mes1Δ arrest) is evident in sgo1Δ cells. c.) The Rec8-GFP signal was observed in the indicated cells at late anaphase I (n>30) and at prometaphase II (n>100), and the frequency of centromeric Rec8-GFP displayed in the cells was counted. The spindles were visualized by expressing CFP-Atb2 (α2-tubulin) (Curr Biol 11, 836-45(2001)). d.) Rec8-GFP levels throughout the indicated chromosome sites in the arrested cells were measured prior to meiosis I (mei4Δ arrest) by ChIP assay with the use of anti-GFP antibodies. The bottom panel shows Schizosaccharomyces pombe chromosome I schematically, and the primers (cnt, imr, dg, dh, lys1, mes1) were used there.

Figure 4 is a set of pictures showing that Sgo1 of the present invention localizes at pericentromeric regions during meiosis I. a.) Synchronous meiosis of diploid pat1-114/pat1-114 cell strains (Embo J 22, 5643-53(2003)) was sampled, meiotic nuclear division was monitored by DAPI staining, and the protein level of Sgo1 was detected by Western blotting with the use of anti-Sgo1 antibodies. b.) Sgo1 (green) was counterstained with tubulin (red) and DAPI (4'6'-diamidino-2-phenylindole) (blue) at the indicated stages in meiotic cells. c.) A sgo1+-GFP cell co-expressing mis6+-CFP was examined under fluorescence microscopy. Sgo1-GFP (green) and Mis6-CFP (red) are merged. d.) Sgo1-GFP levels throughout the indicated chromosome sites in cells arrested at metaphase I were measured by ChIP assay with the use of anti-GFP antibodies. The same primers as for Fig. 2d in synchronism with additional primers at mat (heterochromatin region at the mating type locus) and TAS (telomere associated sequence) were used. e.) Sgo1-GFP (green) was detected at

metaphase I in the indicated cells that express CFP Atb2 to visualize spindles (red). f.) Rec8-HA was expressed with or without Sgo1-FLAG in proliferating cells, and the extracts were immunoprecipitated with anti-FLAG antibody. g.) A model for the action of shugoshin in meiosis. Shugoshin protects centromeric Rec8 complexes from cleaving by separase at the onset of anaphase I, thereby preserves the centromeric cohesion until meiosis II. Shugoshin is degraded depending on APC during anaphase I.

Figure 5 is a set of pictures showing the time-dependent change of the expression levels of Sgo1 and Rec8 in synchronous culture of haploid pat1-114 cell strains (wt), and of cut1-206 or Prad21-slp1 cells. The expression of slp1+ (a fission yeast CDC20 homologue required for APC activation (Mol Cell Biol 17, 742-50(1997))) was repressed during meiosis in Prad21-slp1 cells where slp1 promoter was replaced with rad21. Meiotic nuclear division was monitored by DAPI staining, and the protein levels of Sgo1, Rec8, and tubulin (control) were measured by western blotting with the use of anti-Sgo1, anti-Rec8 and anti-tubulin antibodies, respectively. Although cut1-206 cells together with normal kinetics led to Sgo1 degradation, Rec8 degradation was delayed. Prad21-slp1 cells showed delayed degradation of Sgo1 as well as Rec8. Arrowheads indicate a cleavage product of Rec8 by separase Cut1.

Figure 6 is a set of pictures showing that ectopic expression of sgo1+ inhibits the growth of the cut1-206 mutant. Chromosomal sgo1+ promoter was replaced with Pnmt1 or Pnmt41 (a weaker version of Pnmt1), and the effect on the mitotic growth in cut1-206 temperature-sensitive cells was examined. The indicated cells were streaked on a plate without thiamine and

cultured for 3 days at 28°C. The cut1-206 cells moderately expressing Sgo1 by Pnmt1, arrested mitotic growth even at the permissive temperature, whereas cut1+ cells grew normally.

Figure 7 is a set of pictures showing that Sgo2 of the present invention plays an important role in mitotic at centromere. a.) Serial dilutions of the indicated cultures were spotted onto YEA plates containing 0, 5 or 10 μg/ml of TBZ, and cultured for 3 days at 30°C. b.) The indicated strains were streaked on YEA plates and cultured for 3 days at 30°C. c.) Sgo2-GFP (green) was detected at anaphase I in wild-types and in bub1Δ cells that express CFP-Atb2 to visualize spindles (red). DNA was stained with Hoechst (blue). Wild-type cells at anaphase are also shown. d.) The sgo2+-GFP mis6+-HA cells were fixed and stained with anti-GFP and anti-HA antibodies. e.) Sgo2-GFP levels were measured throughout the indicated chromosome sites in cells arrested at prometaphase or in asynchronous cells by ChIP assay.

Figure 8 is a set of pictures showing the results of analysis of budding yeast shugoshin ScSgo1 of the present invention. a.) Budding yeast ScSGO1-GFP diploids in proliferation were fixed with methanol and counterstained with DAPI. b.) ScSGO1-Myc NDC10-HA cells were fixed, and stained with DAPI and antibodies against Myc and HA. c.) ScSGO1-GFP diploids causing meiosis in culture medium were fixed with methanol and counterstained with DAPI. d.) Serial dilutions of the indicated cultures were spotted onto YPD plates containing 0 or 15 μg/ml of benomyl. e.) Chromosome loss was analyzed in wild-types (wt) and Scsgo1Δ mutants by a colony sectoring assay. The loss of nonessential chromosome fragments resulted in a red sector in a white colony. As a

positive control, ubr1Δ mutant was used (Nature 410, 955-9(2001)). The frequency of sectoring colonies is shown at the bottom (n>120). f.) Samples of segregation of cenV-GFP in Scsgo1Δ tetrads. The segregation patterns in tetrads were mostly classified as one of the three shown at the bottom. The each population (n=200) is also shown. g.) ScSGO1-Myc diploids were induced by synchronous meiosis and were examined the segregation of cenV-GFP marked on one of two homologues at meiosis I and meiosis II. Although most of the cells caused reductional segregation pattern at meiosis I (96%, n=207), the incidence of non-segregation was high at meiosis II (34%, n=322). h.) The cells marked with cenV-GFP on both homologues were induced to meiosis, and counterstained with anti-tubulin antibody and DAPI. Cells at late anaphase I were examined for cenV-GFP dots. ScSGO1-Myc cells frequently showed split cenV-GFP dots at either pair of sister chromatids (72%, n=138), while control wild-type cells did not (<2%, n=106).

Figure 9 is a set of pictures showing sequences of the amino terminal coiled-coil regions and carboxyl terminal basic regions of shugoshin-like proteins in various organisms. The primary sequences of the amino terminal regions of Sgo1 are conserved in Schizosaccharomyoes pombe (Sgo1 and Sgo2), budding yeast (ScSgo1) and Neurospora crassa (B23G1.060), while the sequences containing MEI-S332 in other species are not conserved, all presumably carry coiled-coil motif (predicted by COILS program (Science 252, 1162-4(1991))). See the arrowheads, asterisks and circles in the pictures.

Figure 10 is a picture showing the results of examination of sgo1 mutations that were generated within conserved regions. Both h+sgo1Δ and h-sgo1Δcen2-GFP cells transformed with the

indicated plasmid, were mixed on SPA plates and monitored for segregation of cen2-GFP at miosis II. A plasmid pREP81 bearing a weak version of the thiamine-repressible nmt1 promoter was used to express sgo1. Control cells carrying plasmid pREP81-sgo1 (wt) showed nearly 80% the segregation at meiosis II, whereas cells expressing non-segregation sgo1 allele showed random segregation (50% segregation). Any of the mutations tested, except a non-conserved site mutation 297TA, did not complement sgo1Δ in this assay. The means of two independent experiments are shown (n>100).

Figure 11 (a) is a picture showing schematic representation of the shugoshin family proteins. A predicted coiled-coil (red) and a conserved basic region (blue) exist in the N-terminal and C-terminal regions respectively. Further, figure 11 (b) is a picture showing the result of analysis in HeLa cell extracts by western blotting after transfection with siRNA.

Figure 12 is a set of pictures showing the results that HeLa cells were stained (green) with antibody against hSgo1 or hSgo2 prepared from rabbit, concurrently stained with tubulin antibody and DAPI, and then respectively co-stained with spindle (red) and chromosome DNA (blue). Meanwhile, the cells were fixed with paraformaldehyde.

Figure 13 is a set of pictures showing the results that HeLa cells at prometaphase and metaphase were stained with antibodies against hSgo1 or hSgo2 (green), and concurrently co-stained with antibodies against centromere protein CENP-A (a, c; red), antibodies against passenger protein Aurora B of chromosome localized within kinetochore from prophase to metaphase (b, d; red), and DAPI (blue). Both signals of hSgo1

and hSgo2 showed signals at the sites close to CENP-A dots on chromosome. From the above, it was revealed that both hSgo1 and hSgo2 are centromere proteins. Furthermore, both sites of Sgo1 and Aurora B were practically the same at prometaphase and metaphase, whereas Sgo2 was placed just outside Aurora B. From the above, it was revealed that both hSgo1 and hSgo2 are placed within kinetochore from prometaphase to metaphase.

Figure 14 is a picture showing the results of RNAi experiments that targeted hSgo1 and hSgo2 respectively. The expressions in any proteins were significantly suppressed after 48 hours, thereby the cells arrested in mitosis (total in the figure) were accumulated. As the accumulation was dissolved by suppressing a spindle checkpoint factor BubR1 by RNAi, it was suggested that hSgo1 and hSgo2 directly or indirectly function during the process where spindle take kinetochore properly at centromeres.

Figure 15 is a set of pictures showing the results, where RNAi experiments targeting hSgo1 was performed by using HeLa cells, and then the cells were mounted on a slide glass and stained with Giemsa. It was revealed that sister chromatid strongly adhered at centromere site in control cells; but in cells suppressed hSgo1, the adhesion at centromere site was weak, and easily detached by the experiment operation.

Figure 16 is a set of pictures showing that Sgo1 and Bub1 are required for condensation at centromeres in mitosis. (a) By treatmesnt with siRNA, chromosome spread was performed in mitotic HeLa cells stained with Giemsa. Representative spread is shown together with the occurrence rates. More than one hundred of the prophases and prometaphases were observed for each RNAi. An example of sister chromatid pair is magnified

at the top. (b) After treatment with nocodazole for 4 hours, chromosome spread was observed in cells interfered with RNAi. Examples of the spread are shown with the frequency ($n > 100$). (c) HeLa cells expressing Scc1-myc were fixed at 36 hours after the treatment with siRNAs. The cells were immunostained with anti-myc-antibody (green) and anti-centromere-antibody (ACA) (red). DNA was stained with DAPI (blue). (d) Rates of the cells showing Scc1-myc staining are shown. Cells expressing Scc1-myc in this cell line were less than 25%. Scale bar shows 10μm.

Figure 17 is a set of pictures showing the results of RNAi experiments targeting Bub1, respectively. (A, B) RNAi experiments targeting Bub1 were performed respectively, and resulted in disappearance of the localization of both proteins, hSgo1 and hSgo2 at centromere. (C, D) As the localization of both proteins, hSgo1 and hSgo2 at centromere was normal in RNAi experiments targeting a control, BubR1; the significance of the results of Bub1 was ensured. It is shown that Bub1 and BubR1 are similar but different proteins, and the localization of hSgo1 and hSgo2 at centromere depends on Bub1 (A, B), but not on BubR1 (C, D).

Figure 18 is a set of pictures showing the results that a clone in which cDNA of mouse shugoshin homologous gene (SEQ ID NOs: 21 and 23) is fused with GFP gene was generated by using retroviral vector, and expressed in human HeLa cells. It was revealed that any of the GFP fusion proteins is co-localized with human kinetochore protein Bub1 in mitosis.

Best Mode of Carrying Out the Invention

[0012]

As for a protein of the present invention, a protein Sgo1 (shugoshin) comprising an amino acid sequence shown in SEQ ID NO: 2 and having a regulatory activity of chromosome segregation; a protein comprising the amino acid sequence shown in SEQ ID NO: 2 where one or several amino acids are deleted, replaced or added, and having a regulatory activity of chromosome segregation; a paralogue Sgo2 of protein Sgo1 comprising an amino acid sequence shown in SEQ ID NO: 4 and having a regulatory activity of chromosome segregation; a protein comprising the amino acid sequence shown in SEQ ID NO: 4 where one or several amino acids are deleted, replaced or added, and having a regulatory activity of chromosome segregation; a Saccharomyces cerevisiae homologue ScSgo1 of protein Sgo1 comprising an amino acid sequence shown in SEQ ID NO: 6 and having a regulatory activity of chromosome segregation; a protein comprising the amino acid sequence shown in SEQ ID NO: 6 where one or several amino acids are deleted, replaced or added, and having a regulatory activity of chromosome segregation; a protein (NC) comprising an amino acid sequence shown in SEQ ID NO: 8 and having a Neurospora crassa-derived regulatory activity of chromosome segregation; a protein comprising the amino acid sequence shown in SEQ ID NO: 8 where one or several amino acids are deleted, replaced or added, and having a regulatory activity of chromosome segregation; a protein (At) comprising an amino acid sequence shown in SEQ ID NO: 10 or 12 and having a Arabidopsis-derived regulatory activity of chromosome segregation; a protein comprising the amino acid sequence shown in SEQ ID NO: 10 or 12 where one or several amino acids are deleted, replaced or added, and having a regulatory activity of chromosome segregation; a protein (Mm) comprising

an amino acid sequence shown in SEQ ID NO: 14 or 16 and having a mouse-derived regulatory activity of chromosome segregation; a protein comprising the amino acid sequence shown in SEQ ID NO: 14 or 16 where one or several amino acids are deleted, replaced or added, and having a regulatory activity of chromosome segregation; a protein (Hs) comprising an amino acid sequence shown in SEQ ID NO: 18 or 20 and having a human-derived regulatory activity of chromosome segregation; and a protein comprising the amino acid sequence shown in SEQ ID NO: 18 or 20 where one or several amino acids are deleted, replaced or added, and having a regulatory activity of chromosome segregation; can be exemplified. Further, as for the regulatory activity of chromosome segregation described in the above, although it is not especially limited as long as the activities regulate chromosome segregation, for example, activities correctly regulating chromosome segregation of germ cells and/or of somatic cell division are preferable, and activities protecting (Shugo) the centromere of sister chromatid from the separation in meiosis I is more preferable. In addition, proteins of the present invention can be prepared by known methods based on DNA-sequence information and the like, and the derivations are not limited to yeast, mouse, human and the like. Furthermore, for example, Sgo1 (shugoshin) mutant that is a protein comprising an amino acid sequence shown in SEQ ID NO: 2 where one or several amino acids are deleted, replaced or added, and having a regulatory activity of chromosome segregation, can be prepared by ordinary methods such as known gene manipulation, point mutation and the like. [0013]

As for a DNA of the present invention, a DNA encoding a

protein of the present invention that has a regulatory activity of chromosome segregation: a DNA derived from fission yeast Schizosaccharomyces pombe, comprising a base sequence shown in SEQ ID NO: 1 or 3 or a complementary sequence thereof; and a DNA containing part or whole of these sequences, encoding a protein that has a regulatory activity of chromosome segregation: a DNA derived from Saccharomyces cerevisiae, comprising a base sequence shown in SEQ ID NO: 5 or a complementary sequence thereof; and a DNA containing part or whole of these sequences, encoding a protein that has a regulatory activity of chromosome segregation: a DNA derived from Neurospora crassa, comprising a base sequence shown in SEQ ID NO: 7 or a complementary sequence thereof, and encoding a protein that has a regulatory activity of chromosome segregation; and a DNA containing part or whole of these sequences, encoding a protein that has a regulatory activity of chromosome segregation: a DNA derived from Arabidopsis, comprising a base sequence shown in SEQ ID NO: 9 or 11 or a complementary sequence thereof, and encoding a protein that has a regulatory activity of chromosome segregation; and a DNA containing part or whole of these sequences, encoding a protein that has a regulatory activity of chromosome segregation: a DNA derived from mouse, comprising a base sequence shown in SEQ ID NO: 13 or 15 or a complementary sequence thereof, and encoding a protein that has a regulatory activity of chromosome segregation; and a DNA containing part or whole of these sequences, encoding a protein that has a regulatory activity of chromosome segregation: a DNA derived from human, comprising a base sequence shown in SEQ ID NO: 17 or 19 or a complementary sequence thereof, and encoding a protein that has a regulatory

activity of chromosome segregation; and a DNA containing part or whole of these sequences, encoding a protein that has a regulatory activity of chromosome segregation: a DNA hybridizing with the above DNA under stringent conditions, encoding a protein that has a regulatory activity of chromosome segregation: and the like, can be exemplified.

[0014]

These DNAs can be prepared by known methods based on DNA-sequence information, such as a gene or cDNA library of yeast, mouse, human and the like. Further, using a base sequence shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, or others or a complementary sequence thereof, or part or whole of these sequences as a probe, DNA libraries of yeast, mouse, human and the like are hybridized under stringent conditions, and the intended DNA encoding a protein that has a regulatory activity of chromosome segregation can be obtained by isolating the DNAs that hybridized with the probes. As for a condition of hybridization to obtain the DNA; hybridization at 42°C, and washing treatment by a buffer containing 1× SSC and 0.1% SDS at 42°C; preferably hybridization at 65°C, and washing treatment by a buffer containing 0.1× SSC and 0.1% SDS at 65°C; can be exemplified. Moreover, as for an element affecting the stringency of hybridization, there are various elements other than the above described temperature conditions, those skilled in the art can actualize the stringency equivalent to that of hybridization as exemplified in the above with an appropriate combination of various elements.

[0015]

As for a fusion protein of the present invention, any protein can be used as long as the protein of the present

invention is bound to a marker protein and/or a peptide tag, as for a marker protein, it is not especially limited but a conventionally known marker protein, for example, alkaline phosphatase, Fc region of antibody, HRP, GFP and the like can be exemplified. Further, as for a peptide tag of the present invention, conventionally known peptide tags such as Myc, His, FLAG and GST tags can be specifically exemplified. The fusion protein can be produced by ordinary methods; and is useful for purification of protein Sgo1 and the like by using the affinity of Ni-NTA and His tag, and for a reagent for study in the art.
[0016]

As for an antibody specifically binding to a protein of the present invention, immunospecific antibodies such as monoclonal antibody, polyclonal antibody, chimeric antibody, single-stranded antibody, humanized antibody and the like, can be specifically exemplified. These antibodies can be produced by ordinary methods with the use of proteins such as the above-mentioned Sgo1 or part thereof as an antigen, and among them a monoclonal antibody is preferable in terms of specificity. Antibodies such as a monoclonal antibody are useful for elucidating the localization of Sgo1 and others in vivo.
[0017]

The above-mentioned antibodies of the present invention can be generated with the use of common protocol by administering proteins of the present invention or fragments containing epitope thereof, or cells expressing the protein on their membrane surfaces, to animals (preferably non-human). For example, for preparation of a monoclonal antibody any method such as hybridoma (Nature 256, 495-497, 1975), trioma, human B cell hybridoma (Immunology Today 4, 72, 1983) and EBV-

hybridoma (MONOCLONAL ANTIBODIES AND CANCER THERAPY, pp.77-96, Alan R.Liss, Inc., 1985), by which antibodies are generated from cultures of continuous cell lines, can be used.

[0018]

To generate a single-stranded antibody against a protein of the present invention, a method for preparation of single-stranded antibody (U.S. patent No. 4,946,778) can be applied. Further, to express a humanized antibody, transgenic mouse or other mammals can be used, clones that express a protein of the present invention with the use of the above-mentioned antibody can be isolated/identified, and its polypeptide can be purified by affinity chromatography. Antibodies against peptide containing proteins of the present invention or antigen epitopes thereof can be possibly used for diagnosis and treatment of cancer, or of chromosome segregation diseases such as infertility or Down's syndrome using a regulatory factor of chromosome segregation as an index.

[0019]

Functional analysis of a protein of the present invention can be performed by using fusion proteins fused with, for example; fluorescent substances such as FITC (fluorescein isocyanate) or tetramethyl rhodamine isocyanate; radioisotopes such as 125I, 32P, 14C, 35S or 3H; labelings with enzymes such as alkaline phosphatase, peroxidase, β-galactosidase or phycoerythrin; fluorescence emission proteins such as green fluorescent protein (GFP); or the like, to antibodies such as the above-mentioned monoclonal antibodies. As an immunological assay method with the use of antibody of the present invention, methods such as RIA, ELISA, Fluorescent antibody method, Plaque forming cell assay, Spotting method,

Hemagglutination testing, Ouchterlony method can be exemplified.

[0020]

The present invention will be explained in detail in the following by referring to the examples, but the technical scope of the present invention will not be limited to these.

Example 1

[0021]

[Method]

(Screening of Rec8 protector)

The present inventor examined a gene that is toxic only when co-expressed with Rec8 in vegetative cells. The Rec8 encoding sequence that was fused with GFP was cloned into pREP82 (ura4+ marker) under the thiamine-repressible nmt1+ promoter, to construct pREP82-rec8+-GFP. A Schizosaccharomyoes pombe cDNA library constructed by mRNA that was prepared from meiotic cells, and a pREP3 vector (nmt1+ promoter, LEU2+ marker) (Y.Akiyoshi and Y.W., unpublished) were used. The leu1 ura4-D18 cells carrying pREP82-rec8+-GFP were transformed with the cDNA library, spread on agar plates containing thiamine (promoter-off) and incubated for 3 days at 30°C. The colonies were then replicated on two thiamine-free agar plates: one that contains uracil and 5'-fluoroortoic acid (5'-FOA) where only cells lacked the plasmid pREP82-rec8+-CFP can grow (thereby expresses a library clone alone), and the other that does not contain 5'-FOA (allows co-expression of rec8+-GFP and a library clone). The present inventor added Phloxine B, a drug that stains dead cells red, onto the both agar plates, thereby illuminated sick colonies. After incubation for two days, the

colonies exhibiting sickness only on the co-expression agar plate were picked up, and the library-derived plasmids were recovered and analyzed.

[0022]

(Schizosaccharomyoes pombe strains)

Deletion and tagging of GFP or FLAG to endogenous sgo1+ and sgo2+ were performed by a PCR-based gene targeting method (Yeast 14, 943-951(1998)). By inserting GFP into the C-terminus of the PCR-amplified sgo1+-FLAG, sgo1+-FLAG-GFP was generated and integrated into the endogenous sgo1 locus. Further, a endogenous promoter of the sgo1+ was replaced with a nmt promoter to generate Pnmt-sgo1+ or Pnmt-sgo1+-FLAG-GFP by the PCR-based gene targeting method. The proteins tagged to Sgo1-GFP or Sgo1-FLAG was deleted depending on the purpose. A mei4Δ mutation was used to arrest meiotic cells prior to meiosis I (close to late prophase in meiosis I), and a mes1Δ mutation was used to arrest after meiosis I, as described previously (Nature 400, 461-4(1999)).

[0023]

(Observation of chromosomes marked with GFP)

To observe the segregation patterns of homologues at meiosis I, h90 cells retaining cen2-GFP (Embo J 22, 2284-96(2003)) were spotted on meiosis-inducing medium, SPA. To examine the segregation patterns of sister chromatids, opposite mating type cells, one marked with cen2-GFP and the other not marked, were mixed and spotted on SPA. After incubation for one day, the zygotes were monitored for GFP. Images were obtained under a microscope (Axioplan2, Zeiss) equipped with a cooled CCD camera (Quantix, Photometrics) and by using Metamorph software (Universal Imaging Corporation).

Seven Z-sections for GFP signals were converted to single two-dimensional images by taking the maximum signal at each pixel position in the images.

[0024]

(Chromatin immunoprecipitation; ChIP)

Diploid sgo1+-FLAG-GFP was used for ChIP with Sgo1. To achieve a highly synchronous culture, the endogenous slp1+ promoter was replaced with the rad21+ promoter that is not active during meiosis, and the cells were arrested at metaphase I. The cells were incubated in nitrogen-depleted medium for 17 hours at 30°C, and 60% the cells or less were arrested at metaphase I. For ChIP with Sgo2, nda3-KM311 sgo2+-GFP cells were proliferated at 30°C, and then shifted to 18°C. After incubation for 8 hours, most of the cells were arrested at metaphase. The cells were fixed with 3% para-formaldehyde for 30 minutes at 18°C, and extracts were prepared. The DNA was broken to an average size of 400 bp, and the extracts were immunoprecipitated with rabbit anti-GFP antibodies (Clontech). DNAs prepared from the whole cell crude extracts, or immunoprecipitated chromatin fractions were analyzed by quantitative PCR, with a LightCycler or a Lightcycler-DNA Master SYBR Green I kit (Roche Molecular Biochemicals). Antibody-minus samples were used as controls in each experiment to explain the nonspecific binding in the ChIP fractions.

[0025]

(Preparation of anti-Sgo1 antibodies)

Sgo1+ ORF was PCR-amplified from an Schizosaccharomyces pombe cDNA library, and inserted into plasmids pGEX4T-2 (Pharmacia Biotech) and pET-19b (Novagen) respectively to prepare recombinant proteins GST-Sgo1 and His-Sgo1. GST-Sgo1

was used to immunize rabbit, and the raised antibodies were purified by His-Sgo1 as described previously (Embo J 22, 5643 − 53(2003)). Furthermore, for the purpose of analyzing proteins (SEQ ID NOs: 18 and 20; hSgo1 and hSgo2 respectively) encoding human shugoshin homologous gene (SEQ ID NOs: 17 and 19), part of hSgo1 and hSgo2 was expressed in E coli, and antibodies against hSgo1 and hSgo2 were produced by injecting the protein into rabbit.

[0026]

(Immunostaining)

To stain endogenous Sgo1, wild-type diploid cells cultured for 5 hours in MM-N were fixed with 3% formaldehyde for 40 min at 30°C, and stained by the method described previously (Embo J 22, 5643 − 53 (2003)). To stain Sgo2-GFP and Mis6-HA, logarithmically growing cells were used. Sgo1 was detected by using rabbit anti-Sgo1 antibody at 1:50 and Alexa488-conjugated anti-rabbit antibody (Molecular Probes) at 1:100. Tubulin was detected by using mouse anti-tubulin antibody TAT-1 (provided by Keith Gull) at 1:200 and Cy3-tagged anti-mouse antibody (Chemicon) at 1:2000. Cells were counterstained with DAPI to visualize DNA. The Sgo2-GFP was detected by using mouse anti-GFP antibody (Roche) at 1:50 and BODIPY FL-conjugated anti-mouse antibody (Molecular Probes) at 1:100. The Mis6-HA was detected by using rabbit anti-HA antibody Y-11 (Santa Cruz) at 1:50 and Alexa488-conjugated anti-rabbit antibody at 1:100. Cells were counterstained with DAPI to visualize DNA. Further, immunostaining was performed by using rabbit anti-hSgo1 antibody and rabbit anti-hSgo2 antibody in the same manner as the above.

[0027]

(Coimmunoprecipitation)

Padh-rec8+-3HA Pnmt41-sgo1+-FLAG-GFP strain cells and control Padh-rec8+-3HA strain cells were cultured without thiamine for 15 hours at 30°C, collected, and the extracts were prepared. To liberate chromatin-bound proteins, the extracts were treated with DNase I. After clarifying the extracts by centrifugation, the Sgo1-FLAG-GFP protein was immunoprecipitated with anti-FLAG antibody M2 (Sigma). The Rec8-3HA and Sgo1-FLAG-GFP were detected by anti-HA antibody Y-11 and anti-FLAG antibody M2, respectively.

[0028]

(Analysis of budding yeast)

All sample strains except those for chromosome loss assay are derivative of SK1 (Cell 98, 91-103(1999)). The chromosome loss assay was performed as described previously (Nature 410, 955−9(2001)). The ScSGO1 gene was deleted or epitope-tagged by using PCR generated cassettes (Yeast 14, 953-961(1998)). Accurate gene targeting was checked by PCR. URA3-GFP dots marking chromosome V (cenV-GFP) were described previously (Cell 98, 91-103(1999)). Sporulation was induced by culturing diploid cells at 30°C as described previously (Dev Cell 4, 535-48(2003)). In situ immunofluorescence was performed as described previously (Dev Cell 4, 535-48(2003)).

[0029]

(Cell culture)

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum and 0.03% L-Glutamine. The HeLa cell strain expressing Scc1-myc was cultured with 200μg/ml of G418 (Invitrogen) and 100μg/ml of Hygromycin B (Wako). Expression of Scc1-myc was induced by incubation with 2μg/ml of Doxycyclin

(Sigma) for 48 hours.

[0030]

(Preparation of anti-human Sgo antibody)

As the information for N-terminal amino acid sequence of human Sgo1 was not obtained from the databases, the present inventor cloned a cDNA fragment that was amplified from a cDNA library (BD Biosciences) with the use of primers recognizing the cloning site of λTriplEx: CTCGGGAAGCGCGCCATTGTG (SEQ ID NO: 38) and the DNA sequence corresponding to the numbers 237-242 in amino acid sequence of Q9BVA8: CCTGGCTGAATCAGCTTTGGTG (SEQ ID NO: 39). The Sequencing revealed that the Sgo1 mRNA encodes a protein having 527 amino acids. To obtain polyclonal antibodies against Sgo1, a cDNA fragment encoding the numbers 109-491 in amino acid sequence of Sgo1 was amplified and inserted into the reading frames of plasmids pGEX4T-2 (Amersham) and pET19b (Novagen) to produce GST-Sgo1 and His-Sgo1 respectively, and followed by immunization of a rabbit (QIAGEN) (performed according to the manufacturer's instructions). His-Sgo1 was affinity-purified on CNBr-activated sepharose (Amersham). Antibodies against Sgo2 were raised with GST-Sgo2 (amino acid numbers 331-631) and purified with His-Sgo2 in the same manner as the above.

[0031]

(Immunofluorescence microscopy and chromosome spreading)

Immunofluorescent staining was performed as described in the above, by using anti-human Sgo1 (1:1000), anti-human Sgo2 antiserum (1:10000), anti-Bub1 (1:1000, MBL), anti-BubR1 (1:1000, MBL), anti-CENP-A (1:1000, MBL), anti-Aurora B AIM-1 (1:1000, BD Biosciences) and anti-tubulin DM1A (1:1000, Sigma). Immunostaining of Scc1-myc was performed as described in the

above, by using anti-myc CM-100 (1:1000, Gramsch Laboratories) and ACA (1:1000, provided from Dr. Yoshinari Takasaki). As a secondary antibody, Alexa Fluor 488 goat anti-rabbit antibody (1:1000, Molecular Probes), Cy3 conjugated anti-mouse antibody (1:1000, CHEMICON), and Cy3 conjugated donkey anti-human antibody (1:1000, Jackson ImmunoResearch Laboratories. Inc) were used. 3 μg/ml of Hoechst 33342 or 0.5 μg/ml of DAPI were used for counter staining. Images were taken by using SlideBook or MetaMorph software.

[0032]

(Chromosome spreading)

HeLa cells during mitosis were collected by mitotic shake-off and incubated with 330nM of nocodazole for 0 up to 4 hours. Chromosome spreading was performed as described in the above.

[0033]

(Immunoblotting)

HeLa cells were boiled with the sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P membrane (Millipore), followed by blocking with 5% Skim milk (Nacalai) in TBST (150mM of NaCl, 20mM of Tris-HCl pH7.4, 0.05% Tween-20). Antibody incubations were performed in 0.1% skim milk TBST supplemented with anti-Sgo1 antibody (1:1000), anti-Sgo2 antibody (1:1000), anti-Bub1 antibody (1:500) and anti-tubulin antibody (1:3000). Blots were produced by ECL (Amersham).

[0034]

(RNAi)

As a siRNA target sequence, hSgo1: AAGUCUACUGAUAAUGUCUUATT (SEQ ID NO: 40) and hSgo2:

AAGCACUACCACUUUGAAUAATT (SEQ ID NO: 41), and human Sgo1: GUGAGCCUCUGUGAAUCAATT (SEQ ID NO: 42) and human Sgo2: GCUCUCAUGAACAAUAACUTT (SEQ ID NO: 43) were respectively selected on hSgo1RNA or hSgo2RNA. Furthermore, as a siRNA target sequence, GAGUGAUCACGAUUUCUAATT (SEQ ID NO: 44) was selected on other siRNA target sequence, Bub1RNA; siRNA target sequence, AACGGGCAUUUGAAUAUGAAA (SEQ ID NO: 45, see JCS, 117, 1577-1589 (2004)) was selected at 2 sites on a spindle checkpoint factor BubR1 RNA. These sequences were synthesized as double strand, and introduced into cells by using oligofectamine (Invitrogen). Furthermore similarly, when producing HIV vector, HeLa cells were transfected with HIV plasmid vector, pMD.G (VSV-G env expressing plasmid), pMDLg/p.RRE (the third generation packaging plasmid) and pRSV Rev (Rev expressing plasmid) by calcium phosphate method, collected the culture supernatant 48 hours after the transfection, and condensed to use as a virus vector.

Example 2

[0035]

[Results]

(Identification of shugoshin Sgo1 in fission yeast)

The replacement of the mitotic cohesin, Rad21/Scc1, with the meiotic version, Rec8, is a prerequisite for protecting centromeric sister chromatid cohesion through anaphase of meiosis I (Cell 103, 1155－68(2000)、Mol Cell Biol 23, 3965－73(2003)). However, when Rec8 was expressed ectopically during mitosis, Rec8 was localized largely at centromeres but disappeared at anaphase, with sister chromatids segregating to opposite sides (Figs.1c and d). Moreover, the ectopic

expression of non-cleavable Rec8 during mitosis (note that Rec8 is cleaved by separase Cut1 during meiosis (Embo J 22, 5643-53(2003))) resulted in an inability to separate sister chromatids (see Fig. 2). Thus, in contrast to the situation during meiosis I, centromeric Rec8 is cleaved by separase during mitosis, and results in separation of sister chromatids. The present inventor thus postulated a meiosis I specific centromeric protector of Rec8 from these observations. To identify this factor, the present inventor searched for a gene that generates toxicity during mitotic growth only when co-expressed with Rec8. This screening identified a novel gene, sgo1+ (ORF: SPBP35G2.03C). The hindrance of growth by Sgo1 was significantly dependent on Rec8, as Sgo1 had little effect on growth when co-expressed with Rad21 (Fig. 1a). Co-expression of rec8+ and sgo1+ resulted in high frequency of the blocked nuclear division, as centromere-associated green fluorescent protein markers (cen2-GFP) segregated to the same side of a septated cell highly frequently (see Figs. b and c). To test the possibility that Sgo1 protects Rec8 from degradation at anaphase, the localization of Rec8 was examined in associated with Sgo1 expression, Rec8 tagged with GFP at its carboxyl terminus was expressed under the control of a constitutive adh1 promoter and induced Sgo1 by using a thiamine-repressible nmt1 promoter. Consequently it was found that the Rec8-GFP signal persisted through anaphase only when Sgo1 was co-expressed (Fig. 1d). As Sgo1 is expressed exclusively in meiosis (DNA micro array data (Nat Genet 32, 143-7(2002)), see below), it was found from the above-mentioned results, that Sgo1 is a protector of Rec8 during meiosis.

[0036]

(Sgo1 protects centromeric cohesion at meiosis I)

To examine whether Sgo1 is actually required for the protection of Rec8 during meiosis, the entire ORF encoding sgo1+ was deleted, and the phenotype was examined. Sgo1Δ cells are viable and showed normal vegetative growth, consistent with the concept that sgo1+ is a meiosis-specific gene. To examine the meiotic chromosome segregation of sgo1Δ cells, centromere-linked sequences were marked with GFP (cen2-GFP) on only one of the two homologues in a zygote, and the segregation of the GFP dots were monitored during meiosis I. It was revealed that meiosis I emerged normally in sgo1Δ cells, as sister chromatid pairs generally moved together to the same side of each zygote. Therefore, monopolar attachment was intact (Fig. 3a). Moreover, by marking cen2-GFP on both chromosomes, it was determined that accurate segregation was undergone with homologues at meiosis I (data not shown). However, sister chromatid pairs failed to segregate properly at meiosis II, non-segregation was caused in 50% of the cells or less (Fig. 3a). This value is consistent with random chromosome segregation at meiosis II.

[0037]

To examine centromeric cohesion, cen2-GFP marked on both homologues was monitored in zygotes arrested prior to meiosis II via a mes1Δ mutation. Supporting the above results, sgo1Δ cells frequently showed precocious division of centromeres as split cen2-GFP signals prevailed in the dyad nuclei (Fig. 3b). Finally, it was examined whether protection of Rec8 at centromeres is dependent on Sgo1 by monitoring Rec8-GFP at late anaphase I and prometaphase II. While it is significant that Rec8 signals were centromeric in wild-type cells, the Rec8

singals had largely disappeared from the centromeres at these stages in sgo1Δ cells (Fig. 3c). Although all phenotypes of sgo1Δ cells are reminiscent of heterochromatin-deficient Schizosaccharomyoes pombe, in which Rec8 localization to the pericentromeric regions is decreased and centromeric cohesion is lost during meiosis I, leading to random division at meiosis II (Science 300, 1152-5(2003)). Chromatin binding by Rec8 was examined in cells arrested prior to meiosis I by using a chromatin immunoprecipitation (ChIP) assay. In marked contrast to heterochromatin-deficient cells, Rec8 localization was intact in sgo1Δ cells at the pericentromeric regions as well as all other regions tested. These results suggest that the loss of centromeric Rec8 after meiosis I is caused not by an initial defect in Rec8 localization to centromeres but rather by a defect in the preservation of centromeric Rec8 during meiosis I. The above results indicated that the Cut1 separase becomes active at the onset of anaphase I and cleaves most chromosomal Rec8, leaving only centromeric Rec8 intact (Embo J 22, 5643-53(2003)). These results indicated that Sgo1 plays an essential role in protecting centromeric cohesion throughout meiosis I by protecting cohesin Rec8 from separase cleavage.

[0038]

(Sgo1 localizes at centromeres during meiosis I)

To detect the Sgo1 protein, Sgo1-specific antibodies were produced, and the results of Western blotting indicated that Sgo1 is expressed only around at meiosis I (Fig. 4a). The results of immunofluorescence microscopy on cells at various stages of meiosis revealed that Sgo1 appears at late prophase of meiosis I and is fully localized as several punctuate dots

by the point of metaphase I (Fig. 4b). These dots were co-localized with the Mis6 kinetochore protein (Cell 90, 131-143(1997)), and indicated that Sgo1 is a centromere-associating protein (Fig. 4c). At the onset of anaphase I, Sgo1 signals decrease dramatically. It was found that Sgo1 remains undegraded at centromeres in APC-depleted cells arrested at metaphase I but undergoes normal degradation in separase-defective cells (Fig. 5), and indicated that Sgo1 degradation at anaphase I is regulated more directly by the APC rather than through separase. Although residual Sgo1 signals were detectable at the centromeres in early anaphase I, they disappeared completely by the end of anaphase I (Fig. 4b). This indicates that a substantial amount of Sgo1 is required at the onset of anaphase I when separase is fully activated. However, it is considered that the amounts of Sgo1 required are smaller and smaller as anaphase I progressed. This idea is tenable when the separase activity is quickly down-regulated or when the access to chromosomes is prevented during anaphase I. Sgo1 never reappears during meiosis II (Fig. 4b), and which is consistent with the idea that Sgo1 is required for the protection of Red8 only during meiosis I.

[0039]

The present inventor has already reported that Rec8 localization at pericentromeric regions is especially important for the persistence of centromeric cohesion throughout meiosis I (Science 300, 1152-5(2003)). If Sgo1 is a centromeric protector of Rec8, then it might be expected to localize there as well. To test this possibility, Rec8 localization was delineated more precisely by using the ChIP assay. Sgo1 actually associated with pericentrmeric

heterochromatin regions rather than with central core regions along the centromere sequences (Fig. 4d). As the results of immunoprecipitation experiments indicated that Sgo1 interacts with Rec8 complexes in vivo (Fig. 4f), the protection was carried out through close interaction. Concurrently, these results indicate that Sgo1 resides at pericentromeric regions and acts to protect centromeric Rec8 from the cleavage of separase at anaphase I (Fig. 4d). It was found that the localization of Rec8 does not depend on Sgo1, and vice versa (figure 3d, figure not shown). Actually, the Rec8 and the Sgo1 are in fact independently generated at pericentromeric regions, as for the localization, the Rec8 and the Sgo1 depend on heterochromatin and Bub1 kinase respectively (figure 4e). In contrast, Rec8 and Sgo1 are localized at centromeres in swi6Δ (heterochromatin deficient) and bub1Δ cells respectively (figure 4e). Thus by localizing independently, it can be ensured that Rec8 is protected only at centromeres not along the chromosomal arm regions.

[0040]

Further, it is indicated that shugoshin shields Rec8 physically from the action of separase and counteracts the effects. On this point, even when the strong expression of Sgo1 dose not express Rec8, the mitotic growth was moderately disturbed (figure not shown); and even when the temperature is tolerated for cut1 allele, it was found that cut1 mutant was killed by moderate expression of Sgo1 (fig. 6).

[0041]

(Sgo2 is a mitotic Sgo1 paralogue in fission yeast)

By a conventional BLAST search of genome databases, the present inventor identified Sgo1-like proteins from

Saccharomyces cerevisiae and Neurospora crassa, and indicated that Sgo1 is a conserved protein (see below). In the same search, a Schizosaccharomyces pombe Sgo1 paralogue which the present inventor named Sgo2, was also identified (ORF: SPAC15A10.15). The sgo2+ gene was disrupted, and it was identified that sgo2Δ cells are viable but show sensitivity to the spindle destabilizing drug thiabendazole (TBZ) (Fig. 7a). As sgo1Δ cells never show such a defect, this phenotype is remarkable. To investigate its cellular distribution, the endogenous sgo2+ gene was tagged with GFP. In proliferating cells, Sgo2-GFP was observed as two or three dots in the nucleus (Fig. 7d). However, Sgo2-GFP co-localized with the centromere protein Mis6 at metaphase and disappeared during anaphase (Figs. 7c and d). The results of ChIP assays showed that Sgo2 chromatin association is detectable only on synchronous populations of mitotic cells, and that chromatin association is localized to the pericentromeric regions (Fig. 7e). By enhancing this localization, sgo2 deletion confers a dramatic defect to chromosome segregation when the heterochromatin-deficient swi6Δ mutation was bound thereto, however which by itself impairs centromeric function slightly (Science 269, 1429-31(1995)) (Fig. 7b). These results indicate that Sgo2 cooperates with centromeric heterochromatin factors to ensure chromosome segregation at mitosis. Moreover, it was found that sgo2Δ cells have a modest increase (up to 15%) in non-segregation of homologues at meiosis I, and indicated that Sgo2 is also important for promoting proper meiosis I. However, the role of Sgo2 does not overlap with that of Sgo1, as sgo1Δ neither causes an apparent defect at meiosis I (Fig. 3a) nor enhances a defect of sgo2 in meiosis.

[0042]

(Shugoshin localization controlled by Bub1)

As centromeric Rec8 cannot be detected after meiosis I in fission yeast bub1 mutants, a conserved centromere-associated kinase Bub1 is considered to function in protecting Rec8 during meiosis, (Nat Cell Biol 3, 522-6(2001)) (Fig. 3c). Although bub1 mutation has pleiotropic effects in meiotic chromosome segregation, it is considered that Sgo1 function can be targeted by Bub1 activity. To elucidate this problem, Sgo1-GFP signals were examined in bub1Δ cells undergoing meiosis. Obviously, Bub1Δ cells were almost completely devoid of accurate centromeric Sgo1-GFP signals, instead showed a diffuse fluorescence in the nucleus (Fig. 4e). Similar results were obtained by using the bub1-K762R point mutation that abolishes the kinase activity (Embo J 22, 1075-87(2003)). Although substantial levels of Sgo1 protein were detected in meiotic bub1Δ cells by Western blot analysis (figure not shown), Bub1 dose not influence protein stability of Sgo1. Thus, the kinase activity of Bub1 is required for incorporating Sgo1 to centromeres, and the observed defects in centromeric protection in bub1Δ cells can be explained by impaired localization of Sgo1.

[0043]

In parallel experiments, it was identified that mitotic Sgo2 localization at centromeres was similarly disturbed in bub1 mutants (Fig. 7c). It has been indicated that loss of Bub1 function causes centromeric function to be weakened (J Cell Biol 143, 1775-87(1998)). In this regard, the bub1-K762R mutation shows co-lethality with swi6Δ, a mutation that also slightly impairs centromeric function via its role in pericentromeric

heterochromatin formation. It was found that sgo2Δ similarly shows co-lethality with swi6Δ (Fig. 7b), and exhibits severe miss-segregation of chromosomes at mitosis (figure not shown). As the sgo2Δ bub1Δ double mutant showed no cumulative defects at all in growth or TBZ sensitivity (Fig. 7a), Sgo2 and Bub1 tandem function was confirmed to ensure chromosome segregation in mitosis by these genetic analyses. Taken all together, the above results revealed that the incorporation of Sgo1 and Sgo2 to centromeres is a crucial function of Bub1 kinase in meiosis and mitosis, respectively.

[0044]

(Characteristics of a budding yeast Sgo1 homologue)

The present inventor identified a single Sgo1 homologue, ScSgo1 in budding yeast (ORF: YOR073W), which has so far not been analyzed. The cellular localization of ScSgo1 was examined by tagging endogenous ScSGO1 with GFP. ScSgo1-GFP was detected mainly as a single dot in proliferating cells, but only in a limited subset of the population (Fig. 8a). Scsgo1-GFP was not detected during the G1/S period (i.e. in cells with no bud or a small bud) but appeared as a dot in G2/M (cells with a large bud and a single nucleus) and disappeared at anaphase (cells with a large bud and a stretched nucleus) (Fig. 8a). The dot is co-localized with Ndc10 kinetochore protein (Fig. 8b). During meiosis, ScSgo1-GFP was detected at the kinetochore only at metaphase I, but never during anaphase I or meiosis II (Fig. 8c). Thus, the pattern of ScSgo1 localization closely resembles that of SpSgo2 in mitosis and SpSgo1 in meiosis.

[0045]

The ScSGO1 gene was disrupted to examine the function of ScSgo1. Although the Scsgo1Δ cells were viable, they grew

slowly and showed sensitivity to the spindle destabilizing drug benomyl (Fig. 8d), and indicated that centromeric function might be impaired. And then the chromosome loss rates in Scsgo1Δ cells were compared with those in wild-type cells by a colony sectoring assay. Whereas 40% of the Scsgo1Δ colonies contained red sectors (which indicate chromosome loss), less than 2% wild-type colonies contained such sectors (Fig. 8e). It was concluded that ScSgo1 plays a crucial role at centromeres to ensure mitotic chromosome segregation. At the onset of meiosis, Scsgo1Δ cells showed significant defects that many cells are arrested with a single nucleus in the meiotic condition. However, among the leaked tetranucleate products of meiosis, the distribution pattern of cenV-GFP was consistent with proper segregation at meiosis I with the exception of random segregation at meiosis II (Fig. 8f). It was also found that tagging chromosomal ScSGO1 with 13Myc at its carboxyl terminus, which by itself causes no detectable defects in mitotic growth or meiosis I, resulted in impaired segregation at meiosis II (34% non-segregation indicates 68% random segregation)(Fig. 8g). Moreover, the ScSGO1-Myc cells showed frequent separation of sister centromeres at late meiotic anaphase I (Fig. 8h), indicated that centromeric cohesion was not properly protected. Concurrently, these results support the idea that ScSgo1 plays a crucial role in protecting centromeric cohesion throughout meiosis I, and meiosis II was ensured thereby as is the case with fission yeast Sgo1.

[0046]

(Conservation of Shugoshin among eukaryotes)

BLAST searches identified only three Sgo1-like proteins, which were all in fungi: Schizosaccharomyoes pombe Sgo2,

Saccharomyces cerevisiae ScSgo1, and Neurospora crassa B23G1.060. As the two conserved regions were found in these proteins, the related proteins are searched under conditions of two block sequences by the BLOCK MAKER and MAST programs (Nucleic Acids Res 26,309-12(1998), Bioinformatics 14, 48-54(1998)). This approach extracted several candidate proteins from various eukaryotes including fly, worm, plant, mouse and human (see SEQ ID Nos: 21-37; drosophila Dm, Ce, Arabidopsis At, mouse Mm and human Hs, respectively, in Fig.9). Especially, this list includes Drosophila MEI-S332, which is previously characterized as a protein essential for preserving centromeric cohesion in meiosis (Cell 83, 247-256(1995)), although the similarity score is marginal (E-value=10). All other proteins in the list show a short stretch of similarity in the carboxyl terminal basic regions, while the primary sequences in the first block are not conserved except that they all contain a putative coiled-coil. The space and sequences between these two blocks diverge among the proteins. As these blocks were previously identified to be important for MEI-S332 function (Genes Dev. 12, 3843-3856(1998)), the importance of the conserved regions in Sgo1 was investigated. Several amino acids were changed individually to alanines in these similarity blocks and the function of the mutant proteins in vivo was examined (Fig. 10). It was found that three conserved amino acids known to be important for MEI-S332 function were also required for Sgo1 function (13N, 34V and 368S in MEI-S332; 29N, 501 and 294S in Sgo1) (marked as arrowheads in Fig. 9). Further, other conserved amino acids in the second block (293P, 296R, 298K, 299L and 300R in Sgo1) were also all required for Sgo1 function (asterisks in Fig. 9), and non-conserved residue 297T

could be changed to alanine without impairing function (circle in Fig. 9). These results indicated that the marginal structural similarity observed among Schizosaccharomyoes pombe Sgo1 and other proteins in various eukaryotes is important. Plants and mammals carry two shugoshin-like proteins, suggesting the possibility that the function of shugoshin diverges to complete mitosis and meiosis as in fission yeast. [0047]

(Proteins encoding human shugoshin homologous gene are specifically localized at centromeres in mitosis)

The present inventor previously identified two putative human Sgo proteins, Sgo1 and Sgo2 in the database, although their overall sequence homology to known Sgo proteins in any species other than human is marginal (Fig. 11a). To examine whether these proteins identified in the database are actually human Sgo homologs, the present inventor examined the localization of the proteins. For this end, the present inventor cultured rabbit polyclonal antibodies against recombinant proteins that were produced in bacteria. The obtained Sgo1 antibodies detected an up to 70kD band (predicted molecular weight is 60kD) in the HeLa cell extracts, and the signal was significantly reduced when cells were treated with siRNA that targets Sgo1 mRNA (Fig. 11b). Similarly, Sgo2 antibodies detected an up to 120kD band (predicted molecular weight is 145kD), the signal was reduced in extracts obtained from cells treated with Sgo2 siRNA (Fig. 11b). These data indicate that both Sgo1 and Sgo2 are expressed at least in proliferating HeLa cells. Next, for the purpose of analyzing proteins (SEQ ID NOs: 18 and 20, respectively hSgo1 and hSgo2) encoding human shugoshin homologous gene (SEQ ID NOs: 17 and

19) that was presumed to be human Sgo homologues, part of hSgo1 and hSgo2 was expressed in E. coli, and antibodies against hSgo1 and hSgo2 were produced by injecting the protein into rabbit, HeLa cells were stained with the antibodies and concurrently with tublin antibodies and DAPI, and co-stained with spindle and chromosome DNA respectively, and the expression of hSgo1 and hSgo2 proteins that were both endogeneous in proliferating cells was examined. The results are shown in figure 12. As shown in Figure 12, both signals of hSgo1 and hSgo2 were also observed as dots on chromosomes from prometaphase to metaphase. As a result of the immunostaining, it was identified that both proteins, hSgo1 and hSgo2 are specifically localized at centromeres at mitotic phase. Further, HeLa cells at prometaphase and metaphase were stained with antibodies against hSgo1 or hSgo2; concurrently co-stained with antibodies against centromere protein CENP-A, and DAPI; and examined the expression of hSgo1 and hSgo2 proteins. The results are shown in figure 13. As shown in Figure 13, both signals of hSgo1 and hSgo2 were observed at sites close to CENP-A dots on chromosomes. As a result of the above, it was revealed that both hSgo1 and hSgo2 are centromere proteins. Further, to examine this possibility, Aurora B, which is a passenger protein of chromosome known to be localized within kinetochore from prophase to metaphase, was stained. The sites of Sgo1 and Aurora B were practically the same at prometaphase and metaphase, whereas Sgo2 was placed just outside Aurora B (see Fig. 13). As a result of the above, it was revealed that both hSgo1 and hSgo2 are placed within kinetochores from prometaphase to metaphase. Representative views of sister kinetochore are magnified on the right. Scale bar is 10μm.

[0048]

(Proteins encoding human shugoshin homologous gene are specifically localized at centromeres in mitosis and play an important role to progress chromosome segregation)

RNAi experiments targeting hSgo1 and hSgo2 were performed respectively. The results are shown in figure 14. As a result, the expressions in any proteins were significantly suppressed 48 hours later, the cells arrested in mitosis (total, in figure) were accumulated as indicated in figure 14. As described above, it was strongly suggested that any protein localized at centromeres in mitosis plays an important role for progressing chromosome segregation. As the accumulation was dissolved by suppressing a spindle checkpoint factor BubR1 by RNAi, it was suggested that hSgo1 and hSgo2 are directly or indirectly function during the process where spindle properly takes the kinetochore at centromeres as described below.

[0049]

Further, the cells for which RNAi experiments targeting hSgo1 was performed by using HeLa cells were mounted on a slide glass and stained with Giemsa. The results are shown in figure 15. It was revealed that sister chromatid at prophase strongly adhered at centromere site in control cells where RNAi was not performed; while in cells suppressing hSgo1 expression, where RNAi was performed, the adhesion was weak at centromere site, and easily detached. Consequently, it was demonstrated that hSgo1 has an important role to maintain the strong cohesion at centromere site in mitosis in proliferating cells. Mitotic cells where Sgo1 protein knockdown was performed by RNAi experiments were collected, and the chromosomes were spread to observe chromosome structure directly. In control cells,

sister chromatids were resolved along the arm regions but showed the primary constriction at centromeres (Fig. 16a i). Amazingly, in Sgo1-depleted cells, sister chromatids were often separated along the whole chromosome length (Fig. 16a iii). In samples where sister chromatids stayed densely close, although sister chromatids did not indicate the primary constriction (Fig. 16a iv), this suggests that centromeric cohesion was lost selectively. Nocodazole treatment activates the spindle checkpoint; thereby the cell cycle is arrested at prometaphase. Such prolonged arrest in M phase causes the complete separation of the connectivity from the chromosomal arm regions. For this reason, sister chromatids are only connected at centromeres, and form 'X shaped' chromosome (Fig. 16b, control). As expected, nocodazole-treatment caused the complete separation of sister chromatids along the chromosome length in Sgo1 RNAi cells (up to 97%) (Figs. 16c and d). Consequently, it was demonstrated that hSgo1 plays an important role to maintain the strong cohesion at chromosomal centromere site in mitosis in proliferating cells.

[0050]

RNAi experiments targeting Bub1 were performed respectively. The results are shown in figure 17. Consequently, the localization of either protein of the hSgo1 and hSgo2 to centromere was disappeared. This result means that the conclusion, "localization of shugoshin to centromere depends on Bub1 kinase", which was found in yeast by the present inventor, is also conserved in higher organisms.

[0051]

Next, clone where cDNA of mouse shugoshin homologous genes (SEQ ID NOs: 21 and 23) was fused with GFP gene was produced

by using retroviral vector and expressed in human HeLa cells. The results are shown in figure 18. Consequently, it was revealed that any of the GFP fusion proteins are also co-localized with human kinetochore protein Bub1 in mitosis. [0052]

The analysis of the above hSgo1 and hSgo2 and the analysis results obtained with the use of mouse shugoshin homologous genes were strongly suggested that shugoshin-like protein in animal cells, which were predicted from the sequence, also have functional conservation with yeast shugoshin.

Industrial Applicability

[0053]

Shugoshin of the present invention that is a regulatory factor of chromosome segregation widely conserved in eukaryotic cells, can be advantageously used for studies on the induction mechanism of cancer in somatic division, the chromosome segregation diseases such as infertility or Down's syndrome in meiotic division, and the like besides on the elucidation of mechanism in chromosome segregation.